Broadband CARS Microscopy for Biomaterials

Feature

By Marcus T. Cicerone and Tak W. Kee, Polymers Division, National Institute of Standards and Technology

The task of tracking very complex processes with high spatial resolution, sensitivity, and chemical specificity in living cells is a major challenge in biological microscopy. Chemical specificity is typically achieved through some form of labeling, which has potential to be somewhat invasive. Raman or infrared (IR) microscopy can be utilized to image samples in their natural form using molecular vibrations as a contrast mechanism. The spatial resolution that can be achieved with IR microscopy is insufficient to resolve cellular components. The use of IR microscopy is further limited by interference of water in the vibrational spectra. Spontaneous Raman microscopy can be performed with high spatial resolution, but suffers from low scattering cross-sections, so that high laser power is often required, introducing the possibility of sample photodamage, especially to live cells. Coherent Anti-Stokes Raman Scattering (CARS) can be performed at significantly lower light levels than spontaneous Raman scattering, increasing the likelihood that CARS can be applied to live cells without inflicting photodamage. This, in addition to the high spatial resolution inherent in nonlinear optical microscopy, has led CARS microscopy to begin emerging as a powerful, noninvasive technique for biological and materials imaging. Examples of potential applications for CARS microscopy are noninvasive monitoring of cell metabolic state and detection of cell signaling response to external stimuli.

The initial work on CARS microscopy dates back to 1982, when it was used to resolve detailed structure of onion-skin cells.[1] In 1999, CARS microscopy was used in a collinear geometry and since then significant progress has been made, including imaging of live cells and photoresist materials.[2] Today, there are two general directions in which CARS microscopy is being taken. One direction involves generation and detection of signal in a narrow spectral band to achieve noninvasive video rate imaging, usually of biological specimens. The other direction is towards hyperspectral imaging. Generation and detection of signal in a broad spectral range provides for high chemical resolving power, but at a slower rate of image acquisition. Until recently, a bandwidth of ~200 cm^{-1} had been achieved and CARS microscopy had been used to image and distinguish two distinct chemical species simultaneously, providing a limited degree of chemical specificity.[3,4] We recently demonstrated a CARS microscope that exhibited an increased breadth of spectral sensitivity (~2500 cm^{-1}), covering the Raman fingerprint region (800 cm^{-1} to 1800 cm^{-1}) and beyond.[5] This approach will allow for chemical identification of multiple species, and enables the continuous monitoring of subtle changes in complex systems, such as biological cells.

Figure 1 is ladder diagram that describes the CARS process. CARS microscopy utilizes pulsed laser light of two frequencies to detect molecular vibrations. Pump light, with a frequency of ω_p, is mixed with Stokes light at ω_s in the sample. When the frequency difference ($\omega_p - \omega_s$) is resonant with a vibrational state in the sample, that state gets coherently populated. Population within the vibrational state is further promoted to a virtual state by a second absorption of pump light at ω_p, and relaxation from this final virtual state produces the anti-Stokes light at $2\omega_p$-ω_s. Because the anti-Stokes light is of shorter wavelength than the pump or Stokes light, CARS avoids the usual problem of contamination from fluorescence. On the other hand, a nonresonant background, which arises from permutations of the absorption and emission processes depicted in Figure 1, presents a spectral contamination, although of lesser magnitude than is typically observed with fluorescence in spontaneous Raman scattering.

Figure 2a is a CARS spectrum of benzonitrile obtained with a broadband CARS microscope, the details of which we have recently reported.[5] With this instrument we can acquire spectra in the range of 500 cm^{-1} to 3100 cm^{-1} with good spatial resolution and relatively fast pixilation rates (17 ms signal acquisition). The broadband CARS spectra are generated by using broadband Stokes light, as indicated by the dashed vertical arrows in Figure 1. The instrument utilizes a single 150-fs unamplified Ti:sapphire laser. The laser output was divided into two parts: one was used as pump (ω_p), and another was focused into a tapered silica fiber to generate broadband Stokes light (ω_s). Use of a single laser practically eliminates temporal jitter between pump and Stokes light. The pump and Stokes light, having powers of 13 mW and 10 mW respectively, were combined in a dichroic beamsplitter

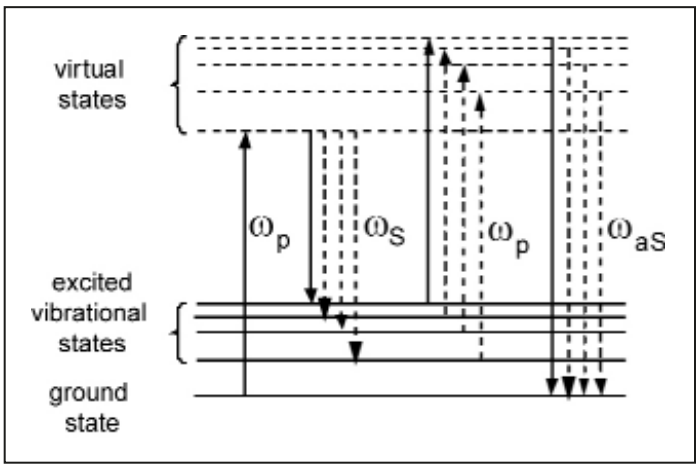

Figure 1. *Energy level diagram for single frequency CARS process (solid vertical arrows) and Broadband CARS (solid and dashed vertical arrows).*

and directed into a 0.8 NA microscope objective. The sample was scanned with a motorized x-y stage and the broadband CARS signal was collected with a 0.5 NA objective. A charge coupled device (CCD) camera mounted on a spectrograph was used for signal detection after spectral filtering. The stability of the spectrum depends highly on the stability of the broadband Stokes light, which has a noise of below 5 percent within an hour. The dashed line in Figure 2a shows the nonresonant background, which accompanies the resonant CARS signal. In Figure 2b, the normalized CARS spectrum of benzonitrile is presented by taking the ratio between the resonant signal to the nonresonant background. Figure 2c is a spontaneous Raman spectrum of benzonitrile acquired in our instrument at a laser power of 23 mW. To contrast the efficiencies of the two processes, the Raman spectrum required 1,000 ms acquisition time to obtain a similar signal-to-noise ratio to the CARS spectrum taken in 17 ms.

The ability of broadband CARS microscopy to perform chemically sensitive hyperspectral imaging is illustrated in Figure 3a. The sample is a tertiary polymer blend containing equal parts

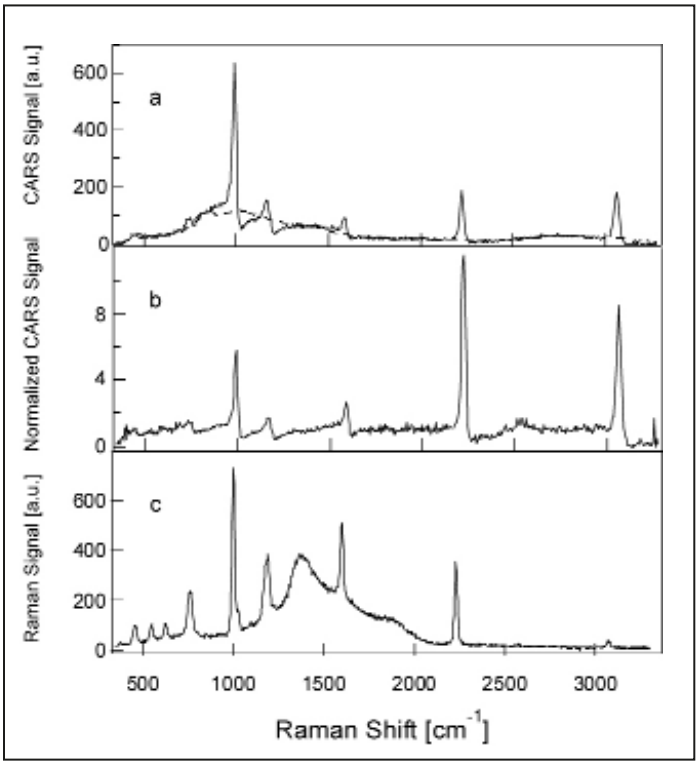

Figure 2. *Broadband CARS spectrum of benzonitrile. Panel a: raw CARS spectrum (solid line), and nonresonant background (dashed line). Panel b: ratio of CARS spectrum to nonresonant background (solid line). Panel c: spontaneous Raman spectrum obtained under identical laser flux (23 mW, but all in pump light).*

of polystyrene (PS), poly(methyl methacrylate) (PMMA) and poly(ethylene terephthalate) (PET). The pseudo colors red, green and yellow in the image are assigned for PS, PET, and PMMA. The image contains 150 x 150 pixels and the dwell time at each pixel is 17 ms. The CARS spectra for each of the components in the blend are given in Figure 3b. The highlighted regions in the spectra are used to assign the identity of a given pixel. By comparing the spectra, we can assign the polymer identity with a confidence level of 99 percent.

We believe that broadband CARS microscopy has notable potential for developing into a widely used imaging technique. In order for this to happen, two primary challenges must be overcome. These are compensation for axial chromatic aberration (ACA) induced by the focusing objective and minimizing the nonresonant background. A poor spatial overlap between the pump and Stokes light due to ACA of the microscope objective can significantly decrease the signal strength. Sensitivity to ACA is a strong function of numerical aperture. Fortunately, at numerical aperture (NA) = 0.8, the requirement that ACA be less than 1 °m over a spectral range of 800 nm to 1,100 nm is not beyond the specifications of commercially available objectives. Thus we were able to use a commercial 0.8 NA objective in our first study.[5] Of course, imaging at higher NA is desirable for increased signal and higher spatial resolution, and the ACA issue will have to be resolved. The presence of the nonresonant background is another major challenge in CARS microscopy because it imposes a lower boundary for detection limit. Reducing or eliminating the nonresonant background is therefore highly desirable. Efforts in addressing these issues are currently underway in our laboratory.

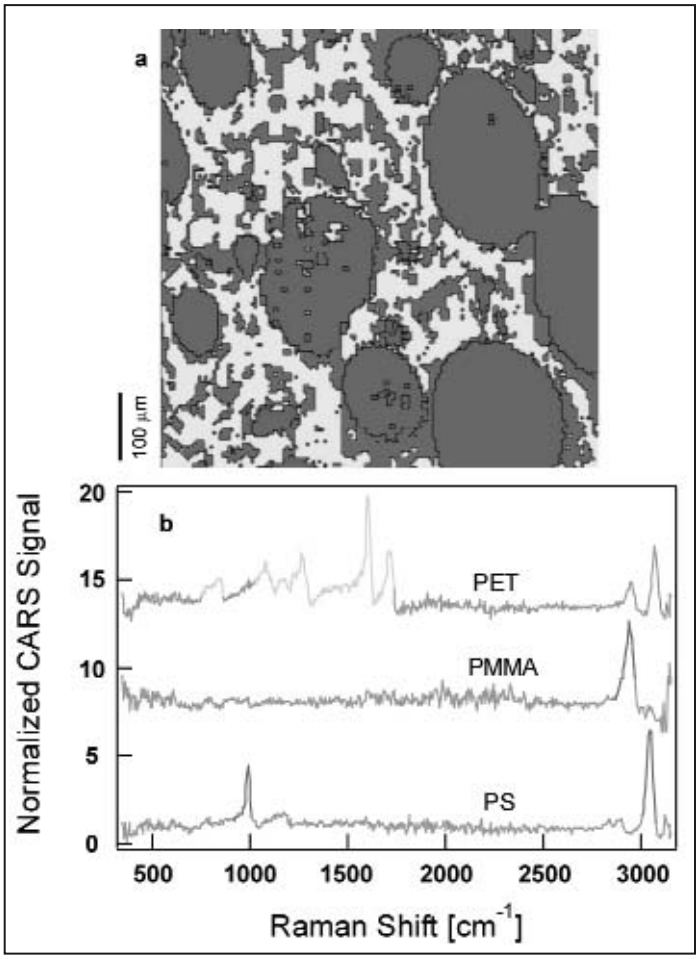

Figure 3. *Panel a: broadband CARS micrograph of a phase-separated polymer blend including equal parts of PMMA, PS, and PET. Panel b: reference spectra from each of the individual polymer components (with arbitrary vertical shift for clarity). The highlighted segments indicate spectral regions that were used for identification of spectra from each pixel in Panel a.*

We are reasonably confident that these issues will be resolved in the near future and an improved sensitivity and signal-to-noise ratio are within reach.

In the past few years, CARS microscopy has been enjoying increased attention and it is continuously generating strong interests in the field of biological imaging. Most recently, Xie et al. have used narrowband CARS microscopy to monitor lipid droplet trafficking in cells and differentiation of fibroblasts to adipocytes.[6] The authors show that before fibroblasts fully differentiate into adipocytes by acquiring a large number of lipid droplets in cytoplasm, there exists a period in which almost all the cytoplasmic lipid droplets disappear. This result has never been observed before with other techniques. This work clearly indicates that the noninvasiveness, high sensitivity and selectivity of CARS present the promise of facilitating increased insights into many more biological processes. We are hopeful that the increase in chemical specificity through hyperspectral

Continued on page 23

BioInk

(Continued from page 21)

Palatin Technologies Inc., Cranbury, N.J., and **King Pharmaceuticals**, Inc., Bristol, Tenn., announced positive results of a phase 2A pilot clinical study evaluating PT-141 in pre-menopausal women diagnosed with female sexual dysfunction (FSD). Patients in the study receiving PT-141 reported increases in their levels of sexual desire and genital arousal compared to placebo. Additionally, there was a correlation between sexual desire and genital arousal in patients receiving PT-141, an observation that further reinforces the potential importance of these reports. Eighteen women with a diagnosis of FSD were enrolled in this double-blind, randomized, placebo-controlled, single-dose, cross-over clinical study. PT-141 is the first compound in a new drug class known as melanocortin receptor agonists under development to treat sexual dysfunction.

St. Jude Medical Inc., St. Paul, Minn., announced the European market launch of the QuickSite® 1056T bipolar left-heart pacing lead, the world's first cardiac resynchronization therapy (CRT) lead to combine bipolar pacing capability with a composite body for superior handling and a unique S-shaped distal tip for outstanding stability. The lead represents the next advance in the QuickSite family of left-heart leads. Specifically designed for placement in the coronary sinus, the St. Jude Medical QuickSite 1056T bipolar lead enables left-ventricular pacing in cardiac resynchronization therapy applications. At 5.5 French, the lead body is as small in diameter as the previously available QuickSite 1056K unipolar lead.

Broadband...

(Continued from page 17)

imaging with our microscope, in addition to the inherently superb spatial and temporal resolution of CARS, will allow the use of broadband CARS microscopy for noninvasively tracking the temporal and spatial course of cellular events involved in complex biological processes such as differentiation and signal transduction, opening windows to insights on biological processes that were closed, or practically closed heretofore.

References

1. Duncan, M. D.; Reintjes, J.; Manuccia, T. J. Opt. Lett. 1982, 7, 350-352.
2. Cheng, J. X.; Xie, X. S. J. Phys. Chem. B 2004, 108, 827-840.
3. Muller, M.; Schins, J. M. Journal of Physical Chemistry B 2002, 106, 3715-3723.
4. Chen, J. X.; Volkmer, A.; Book, L. D.; Xie, X. S. Journal of Physical Chemistry B 2002, 106, 8493-8498.
5. Kee, T. W.; Cicerone, M. T. Opt. Lett. 2004, 29, 2701-2703.
6. Nan, X. L.; Cheng, J. X.; Xie, X. S. J. Lipid Res. 2003, 44, 2202-2208.